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Mammal prolactin variants

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The invention relates to mammal prolactin (PRL) variants, and their use as antagonists of mammal prolactin receptors (PRLRs), more particularly of human prolactin receptor (hPRLR).

5 Prolactin is an anterior pituitary hormone involved in a wide spectrum of biological activities, among which are those related to lactation and reproduction (BOLEFEYSOT *et al.*, *Endocr. Rev.*, 19, 225-268, 1998).

10 PRL actions on target tissues are mediated by a specific membrane-bound receptor, the Prolactin Receptor (PRLR), which belongs to the cytokine receptor superfamily (KELLY *et al.*, *Endocr. Rev.*, 12, 235-251, 1991).

Within the last few years, several studies demonstrated, that PRL is also synthesized in extra-pituitary  
15 sites (for review, see BEN-JONATHAN *et al.*, *Endocr. Rev.*, 17, 639-669, 1996), such as mammary epithelial cells (GINSBURG and VONDERHAAR, *Cancer Res.*, 55, 2591-2595, 1995) or prostate (NEVALAINEN *et al.*, *J. Clin. Invest.*, 99, 618-627, 1997). In addition, it was shown that the hormone exerts a  
20 proliferative action on these cells (expressing the PRLR) via an autocrine/paracrine loop (GINSBURG and VONDERHAAR, *Cancer Res.*, 55, 2591-2595, 1995; MERSHON *et al.*, *Endocrinology*, 136, 3619-3623, 1995; CLEVINGER and PLANK, *J. Mammary Gland. Biol. Neopl.*, 2, 59-68, 1997). Moreover, it has been  
25 suggested that the growth-promoting activity exerted by PRL on some target tissues under normal conditions may be somehow involved in promoting tumor growth under pathological conditions. Experimental evidence supporting this tumor-promoting action of PRL are i) the shortened delay of  
30 appearance of spontaneous breast tumors in PRL-transgenic mice (WENNBO *et al.*, *J. Clin. Invest.*, 100, 2744-2751, 1997), ii) in contrast, the delayed appearance of middle T antigen-induced breast tumors in PRL knockout mice (VOMACHKA *et al.*, *Oncogene*, 19, 1077-1084, 2000), or iii) the extensive  
35 prostate hyperplasia observed in PRL-transgenic mice (WENNBO *et al.*, *Endocrinology*, 138, 4410-4415, 1997).

Due to the failure of clinical treatments using dopamine agonists to reduce breast tumor progression (MANNI

*et al.*, Breast Cancer Res. Treat., 14, 289-298, 1989), PRL has been considered for a long time as a minor player in human breast cancer. However, dopamine agonists fail to target extra-pituitary PRL synthesis, which now appears at least as important as circulating, pituitary-secreted PRL in these phenomena of tumor proliferation. Developing PRLR antagonists able to compete with wild-type prolactin (WT-PRL) for receptor binding, but unable to trigger downstream signalling pathways, appears to be an alternative strategy to prevent, or at least reduce PRL-induced tumor proliferation, with potential implications in pathologies such as breast cancer and prostate hyperplasia (GOFFIN *et al.*, Mol. Cell. Endocrinol., 151, 79-87, 1999). Although analogs of growth hormone (GH) such as G120K-hGH, were reported to antagonize the PRLR (GOFFIN *et al.*, Endocrino., 1999), these analogs also antagonize the GH receptor (GHR). Since this duality of target may be unsuitable in a therapeutic context, development of antagonists specifically targeting the PRLR (and not the GHR) was initiated.

Formerly, the inventors have identified, localized and characterized two binding sites on the hormone, called binding sites 1 and 2, and proposed a model of PRLR activation by sequential homodimerization (GOFFIN *et al.*, Endocr. Rev., 17, 385-410, 1996).

Based on these data, the inventors designed a first generation of human prolactin receptor (hPRLR) antagonists by introducing a sterically hindering mutation within binding site 2 of human PRL (hPRL), thereby preventing this region from docking efficiently with the PRLR molecule (GOFFIN *et al.*, J. Biol. Chem., 271, 16573-16579, 1996). In one of these analogs, referred to as G129R-hPRL, an arginine is substituted for glycine 129 (belonging to site 2), which generates the expected steric hindrance (GOFFIN *et al.*, J. Biol. Chem., 271, 16573-16579, 1996; GOFFIN *et al.*, J. Biol. Chem., 269, 32598-32606, 1994).

The inventors have shown that in some bioassays this hPRL mutant is no longer able to activate the PRLR, presumably because receptor dimerization is impaired; hence,

it acts as an antagonist. These properties were demonstrated first, in a bioassay involving activation of a PRL-responsive luciferase reporter gene by the human or rat PRLR (GOFFIN *et al.*, J. Biol. Chem., 271, 16573-16579, 1996), and second, on proliferation and activation of signalling pathways in various human breast cancer cell lines (LLOVERA *et al.*, Oncogene, 19, 4695-4705, 2000).

However, efficient antagonistic effects required the analog being used in significant molar excess vs. WT-hPRL (10:1 to 50:1) because of its 10-fold lower affinity. In addition, in more sensitive bioassays such as the classical rat Nb2 cell proliferation bioassay, G129R-hPRL failed to exhibit any antagonistic activity (GOFFIN *et al.*, J. Biol. Chem., 269, 32598-32606, 1994) and rather acted as a weak agonist, displaying full activity at higher concentration than hPRL. This residual agonistic activity of G129R-hPRL was confirmed by the inventors *in vitro* using another proliferation assay (Ba/F3 cells transfected with the hPRLR encoding plasmid), and *in vivo* in transgenic mice expressing G129R-hPRL analog: whereas PRLR-deficient mice are sterile and unable to develop a normal mammary gland (ORMANDY *et al.*, Genes Dev., 11, 167-178, 1997), mice expressing G129R-hPRL analog fail to exhibit any reproductive deficiency and lactate successfully, clearly indicating that *in vivo*, G129R-hPRL does not abolish PRL-mediated actions.

These data clearly demonstrate that i) introducing a sterically hindering mutation within binding site 2 (G129R mutation) alters PRL biological properties, which results in antagonistic properties in some homologous (human PRLR-mediated) bioassays, ii) however, this mutation does not completely prevent receptor dimerization, since in more sensitive assays as well as in transgenic mice, the antagonistic properties are taken over by the intrinsic, residual agonistic activity of G129R-hPRL.

Thus, the latter cannot be used therapeutically as a pure antagonist of the prolactin receptor, since it may exert an effect opposite to that expected from an antagonist.

The inventors have undertaken to develop more efficient hPRLR antagonists. They have previously studied the potential involvement of the N-terminal tail of hPRL in its binding to the PRLR. They have engineered iterative N-terminal deletions in hPRL, ranging from removal of the 9 first residues up to the 14 first residues; these mutants are shown on Figure 1.

Legend of Figure 1:

Top: PRL and GH N-terminal sequences are aligned; the N-terminus is 9 residues longer in PRL, including a disulfide bond between Cys<sub>4</sub> and Cys<sub>11</sub>. An arrow identifies putative helix 1 as predicted by homology modeling.

Bottom: incremental deletions of hPRL N-terminus. Deletion of the 9 first residues ( $\Delta$ 1-9-hPRL) mimics N-terminus of hGH, whereas deletion of the 14 first residues ( $\Delta$ 1-14-hPRL) removes the N-terminus tail in its entirety.

They observed that deletion of the 9 first residues of hPRL ( $\Delta$ 1-9-hPRL) slightly enhanced the affinity for the PRLR leading to increased maximal activity compared to wild-type hPRL (WT hPRL) in the luciferase assay, while deletion of the 14 first residues ( $\Delta$ 1-14-hPRL) results in a decrease of the affinity and maximal activity (Endocrine Society 82<sup>nd</sup> Annual Meeting, Toronto, June 21-24 2000, Abstract 613).

The inventors have now undertaken to test the effect of N-terminal deletions on the affinity and antagonistic activity of the G129R-hPRL analog. Therefore, they engineered two N-terminal deletions in G129R-hPRL, by removal of the 9 first residues (mutant  $\Delta$ 1-9-hPRL) and of the 14 first residues (mutant  $\Delta$ 1-14-hPRL).

The inventors found that, unexpectedly, both mutations completely abolished the residual agonist activity of G129R-hPRL.

Without being limited by theory, it may be supposed that these N-terminal deletions impair the formation of the disulfide bridge between Cys<sub>4</sub> and Cys<sub>11</sub>, and that other mutations preventing the formation of said disulfide bridge

may also have advantageous effect on reducing the residual agonist activity.

Accordingly, the present invention provides an antagonist of a mammalian prolactin receptor, wherein said  
5 antagonist is a variant of mammal prolactin having the following mutations:

- a) a mutation or set of mutations within the 14 N-terminal amino acids, wherein said mutation or set of mutations prevents the formation of the disulfide bridge between Cys<sub>4</sub>  
10 and Cys<sub>11</sub>, and
- b) a sterically hindering mutation or set of mutations within binding site 2 of prolactin.

Mutation(s) a) impairing the formation of the Cys<sub>4</sub>-Cys<sub>11</sub> disulfide bridge comprise for instance: deletions  
15 including Cys<sub>4</sub> and/or Cys<sub>11</sub>, or substitution of Cys<sub>4</sub> and/or Cys<sub>11</sub> by an amino acid other than a cysteine.

Mutation(s) b) comprise in particular any substitution of a small amino acid within binding site 2 of PRL by a large and/or charged amino acid in order to  
20 introduce a steric hindrance. Examples of such mutations are for instance substitution of at least one residue among Gln<sub>122</sub>, Leu<sub>125</sub>, Ser<sub>26</sub>, Ala<sub>22</sub> or Gly<sub>129</sub>, preferably Ala<sub>22</sub>, more preferably Gly<sub>129</sub>, by residues such as Tyr, Phe, Asp, Glu, Arg, Lys or Trp, preferably Arg, Lys or Trp.

25 According to a preferred embodiment of the invention, mutation(s) a) comprises the deletion of at least the 4 N-terminal residues, preferably of at least the 9 N-terminal residues of PRL.

In cases wherein the N-terminal deletion is  
30 shorter than 11 amino acids, mutation(s) a) may further comprise the substitution of the Cys<sub>11</sub> residue by an amino acid other than a cysteine. This further allows an easier purification of the variants, by avoiding aggregation thereof that may result from the presence of free SH groups.

35 Preferred PRL variants of the invention are variants comprising the following mutations:



- a deletion of at least the 9 N-terminal residues and up to the 14 N-terminal residues; and
- a G129R substitution.

5           Advantageously, the variants of the invention are variants of human prolactin (hPRL).

          The present invention also provides polynucleotides encoding the PRL variants of the invention.

10           Polynucleotides of the invention may be obtained by the well-known methods of recombinant DNA technology and/or of chemical DNA synthesis. These methods also allow to introduce the desired mutations in a naturally occurring DNA sequence.

15           The invention also provides recombinant DNA constructs comprising a polynucleotide of the invention, such as expression cassettes wherein said polynucleotide is linked to appropriate control sequences allowing the regulation of its transcription and translation in a host cell, and recombinant vectors comprising a polynucleotide or an  
20           expression cassette of the invention.

          These recombinant DNA constructs can be obtained and introduced in host cells by the well-known techniques of recombinant DNA and genetic engineering.

25           The invention also comprises a prokaryotic or eukaryotic host cell transformed by a polynucleotide encoding a PRL variant of the invention.

          A PRL variant of the invention can be obtained by culturing a host cell containing an expression vector comprising a nucleic acid sequence encoding said PRL variant,  
30           under conditions suitable for the expression thereof, and recovering said variant from the host cell culture.

          The invention also provides transgenic non-human animals, in particular transgenic non-human mammals, transformed with a polynucleotide encoding a PRL variant of  
35           the invention. Suitable methods for the preparation of transgenic animals are for instance disclosed in: *Manipulating the Mouse Embryo*, 2<sup>nd</sup> Ed., by HOGAN et al., Cold Spring Harbor Laboratory Press, 1994; *Transgenic Animal*

*Technology*, edited by C. PINKERT, Academic Press Inc., 1994; *Gene Targeting: A Practical Approach*, edited by A.L. JOYNER, Oxford University Press, 1995; *Strategies in Transgenic Animal Science*, edited by G.M. MONASTERSKY and J.M. ROBL, ASM  
5 Press, 1995; *Mouse Genetics: Concepts and Applications*, by Lee M. SILVER, Oxford University Press, 1995.

The invention also relates to a therapeutic composition comprising a PRL variant of the invention, or a polynucleotide encoding said PRL variant, optionally mixed  
10 with suitable carriers and/or excipient(s).

For instance, the PRL variants of the invention can further be conjugated to one or more chemical groups, in order to increase their molecular weight. Examples of suitable chemical groups include polyols, such as  
15 polyethylene glycol (PEG) or heterologous polypeptides preferably hydrosoluble polypeptides, such as serum albumin or fragments thereof.

Therapeutic compositions of the invention are useful as PRLR antagonists, in particular for treating or  
20 preventing diseases involving PRLR-mediated effects, such as tumoral proliferation involving any form of benign or malignant tumor (hyperplasia, dysplasia, neoplasia, adenoma, carcinoma) in any PRL target tissue (breast, prostate, liver, pituitary, lymphocytes), auto-immune diseases (lupus  
25 erythematosus, rheumatoid arthritis), hyperprolactinemia, typically, any diseases arising from an overstimulation of the PRLR (hypermastia, reproduction disorders) (BOLE-FEYSOT et al., Endocr. Rev., 1998).

The therapeutic compositions of the invention can  
30 be administered in various ways:

They can be used systemically or locally. A preferred route of administration is the parenteral route, including for instance intramuscular, subcutaneous, intravenous, intraperitoneal, or local intratumoral  
35 injections.

The oral route can also be used, provided that the composition is in a form suitable for oral

administration, able to protect the active principle from the gastric and intestinal enzymes.

In the case wherein the therapeutic composition includes a polynucleotide encoding a PRL variant of the  
5 Invention, said nucleotide is generally inserted in an expression cassette allowing its expression in a target organ or tissue.

The expression cassette can be directly transferred in the cells as naked DNA, or placed in an  
10 appropriate vector, such as a viral vector, for instance an adenovirus derived vector.

Gene transfer can be performed *ex vivo* on cells removed from the subject to be treated and thereafter re-implanted into said subject, or can be performed by direct  
15 administration of the nucleic acid to said subject.

The choice of the method of transfer and/or of the vector depends on the target organ or tissue, and/or on whether a short-time expression (transient expression) or a more stable expression is wanted.

20 Since the PRL variants of the Invention have a lower affinity for the PRL receptor than native PRL, the amount administered will be chosen in order to supply a large excess of PRL variant over endogenous PRL in the blood and/or target tissue. On the other hand, due to the lack of residual  
25 agonist activity of PRL variants of the invention, high doses thereof can be administered, without risk of unwanted agonist effects. In most of cases, an amount of PRL variant resulting in a 10 to 100-fold excess over endogenous PRL will be suitable. If necessary, an amount of PRL variant resulting in  
30 a 1000-fold excess or more over endogenous can be administered.

The present invention will be further illustrated by the following additional description, which refers to examples illustrating the properties of hPRL antagonists of  
35 the invention. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

# EXAMPLE 1: PRODUCTION AND PURIFICATION OF hPRL ANALOGS

## Hormones

All PRL (wild-type and mutant forms) used in this study were produced by recombinant technology: WT hPRL, the  
 5 binding site 2 analog G129R-hPRL (Gly 129 replaced with Arg), single N-terminal deleted mutants ( $\Delta$ 1-9-hPRL,  $\Delta$ 1-10-hPRL,  $\Delta$ 1-11-hPRL,  $\Delta$ 1-12-hPRL,  $\Delta$ 1-13-hPRL,  $\Delta$ 1-14-hPRL), the double mutants in which mutation G129R was introduced into  $\Delta$ 1-9-hPRL or  $\Delta$ 1-14-hPRL (generating  $\Delta$ 1-9-G129R-hPRL and  $\Delta$ 1-14-G129R-hPRL analogs).  
 10

## Construction of mutated hPRL expression vectors

### N-terminal deletions

#### Constructions

Construction of expression plasmids encoding  $\Delta$ 1-9-hPRL,  $\Delta$ 1-10-hPRL,  $\Delta$ 1-11-hPRL,  $\Delta$ 1-12-hPRL,  $\Delta$ 1-13-hPRL and  $\Delta$ 1-14-hPRL  
 15 analogs was performed using Polymerase Chain Reaction (PCR); plasmid pT7L-hPRL (PARIS et al., Biotechnol. Appl. Biochem., 12, 436-449, 1990) was used as template. Sequences of 5' primers correspond to the 5' sequence of the hPRL cDNA  
 20 lacking the 9 ( $\Delta$ 1-9-hPRL) up to 14 ( $\Delta$ 1-14-hPRL) N-terminal codons. A unique NdeI restriction site (CATATG) containing the ATG codon (methionine initiator) was inserted in the 5' primer. TGC codon encoding Cys 11 was mutated into TCC encoding a serine.

25 The sequence of 5' primers are the following (5' to 3'):

$\Delta$ 1-9: GGCATATGCGATCCCAGGTGACCCTTCG  
 $\Delta$ 1-10: GGCATATGTCCCAGGTGACCCTTCGAG  
 $\Delta$ 1-11: GGCATATGCAGGTGACCCTTCGAGACC  
 $\Delta$ 1-12: GGCATATGGTGACCCTTCGAGACCTGTT  
 30  $\Delta$ 1-13: GGCATATGACCCTTCGAGACCTGTTTG  
 $\Delta$ 1-14: GGCATATGCTTCGAGACCTGTTTGACC

The 3' primer is identical for all analogs; it corresponds to a sequence in the non-coding region of the hPRL cDNA, located in 3' of the unique HindIII restriction  
 35 site: 5'CTGTTACACCCACGCATGG3'.

The PCR reaction was performed as follows: 200  $\mu$ M dNTP; 45  $\mu$ M  $MgCl_2$ , 1.5  $\mu$ l Taq Polymerase (5 u/ $\mu$ l), PCR buffer, 10 ng of template (plasmid pT7L-hPRL), 20 pmoles of each primers. PCR was performed for 25 cycles: 94°C (30 sec), 56°C (30 sec), 72°C (1 min). PCR products were subcloned into TA cloning vector (pCR II.1), then recombinant TA plasmids were digested using *Nde*I and *Hind*III and purified inserts were ligated into pT7L plasmid linearized using identical restriction enzymes. After transformation, *E. coli* BL21(DE3) colonies were analysed for their DNA content; plasmids were extracted and digested to confirm the presence of expected inserts, then sequenced to check the expected mutations.

#### *Production and purification of proteins*

Recombinant WT hPRL and hPRL analogs were overexpressed in a 1 liter culture of *E. coli* BL21(DE3) and purified as previously described (PARIS *et al.*, Biotechnol. Appl. Biochem., 12, 436-449, 1990; GOFFIN *et al.*, Mol. Endocrinol., 6, 1381-1392, 1992). Briefly, when the OD<sub>600</sub> of bacterial cultures reached ~0.9, overexpression was induced using 2 mM isopropylthiogalactoside (IPTG) for 4 h (OD<sub>600</sub> ~2.5 after 4 h). Cell lysis was performed using a cell disintegrator (Basic Z, Cell D, Roquemaure, France). Proteins were overexpressed as insoluble inclusion bodies that were solubilized in 8 M urea (5 min at 55°C, then 2 h at room temperature) and refolded by continuous dialysis (72 h, 4°C) against 50 mM  $NH_4 HCO_3$ , pH 8.

Protein purification was performed using chromatography equipment (GRADIFRAC) and columns (HITRAP Q SEPHAROSE, SEPHACRYL S200 High Resolution) purchased from AMERSHAM-PHARMACIA BIOTECH (Orsay, France).

Two alternative protocols were used. The dialyzed proteins were centrifuged for at least 60 minutes (9000 x g) to remove aggregates before loading the cleared supernatant mixture onto an anion exchange HITRAP Q column (equilibrated in 50 mM  $NH_4 HCO_3$ , pH 8). PRLs eluted in two peaks, one major peak eluted at a concentration of 150 mM NaCl, and a minor one eluted at a higher salt concentration (~200 mM).

Analytical gel filtration of these fractions indicated that the major peak corresponds to monomeric PRL, whereas the minor one includes various multimeric forms. Alternatively, refolded (dialyzed) proteins were concentrated by tangential flow ultrafiltration using a YM10 MINIPLATE bioconcentrator (MILLIPORE CORP.-AMICON, Bedford, MA; 500 ml/min flow rate), then the concentrated solution was centrifuged (10 min, 9000 x g) to remove aggregates formed upon ultrafiltration. Supernatants were purified by gel filtration chromatography using a high resolution SEPHACRYL S-200 column equilibrated in 50 mM  $\text{NH}_4\text{HCO}_3$ , 150 mM NaCl, pH 8. This second protocol usually led to lower yields due to higher protein precipitation upon the ultrafiltration step. Fractions corresponding to monomeric hPRLs (eluted from molecular sieve or anion exchange columns) were pooled, quantified, aliquoted and stored at  $-20^\circ\text{C}$ .

Protein size and purity were assessed using 15% SDS-PAGE under reducing (beta-mercaptoethanol) or non-reducing conditions. Protein fractions were quantified by Bradford protein assay (BIO-RAD Laboratories, Inc., Ivry-sur-Seine, France), using BSA as the reference.

#### Double mutants

Expression plasmids encoding analogs  $\Delta 1-9$ -G129R-hPRL and  $\Delta 1-14$ -G129R-hPRL were constructed by substituting the EcoRI-BglII fragment from pT7L-G129R-hPRL plasmid (containing the G129R mutation) (GOFFIN *et al.*, J. Biol. Chem., 269, 32598-32606, 1994) for the corresponding EcoRI-BglII fragment in pT7L- $\Delta 1-9$ -hPRL and pT7L- $\Delta 1-14$ -hPRL expression vectors. Clones obtained were analysed for the presence of the insert, then sequenced to check the expected mutations. Analog expression using BL21(DE3) bacteria, and protein purification were performed as described above.

All hPRL mutants produced in bacteria as inclusion bodies refolded correctly, suggesting that the various mutations do not disturb global conformation of the protein. This was confirmed by analysis of their content in secondary structure, performed by circular dichroism (not

shown). The only repeated difference between mutated and WT hPRL was that N-terminal deletions tended to increase the monomeric/multimeric ratio observed after protein refolding. It is believed that removal of the two N-terminus cysteines (Cys4-Cys11) prevents formation of covalent multimers responsible for intermolecular disulfide bonding between these residues.

## EXAMPLE 2: AFFINITY OF HPRL ANALOGS FOR HUMAN PRLR

### Binding studies

The affinity of the various hPRL analogs for the human PRLR was estimated by their ability to compete [<sup>125</sup>I]-hPRL for binding to this receptor. Binding affinities were determined using cell homogenates of HL5 cells (expressing the human PRLR), following the procedure previously described (KINET et al., J. Biol. Chem., 274, 26033-26043, 1999).

Briefly, hPRL was iodinated using IODOGEN, and its specific activity was in the range of 40-50 µCi/µg. Binding assays were performed overnight at room temperature using 150-300 µg cell homogenate protein in the presence of 30,000 cpm [<sup>125</sup>I]-hPRL and increasing concentrations of unlabeled competitor (WT or mutated hPRL).

The affinity of WT hPRL for the human PRLR (using HL5 cell homogenates) as calculated by Scatchard analysis indicated a K<sub>d</sub> of 3.4 (± 1.3) × 10<sup>-10</sup> M (KINET et al., J. Biol. Chem., 1999).

### Binding assay of single N-Terminal hPRL mutants.

The relative binding affinity of hPRL analogs was calculated as the ratio of their IC<sub>50</sub> with respect to that of WT hPRL calculated from competition curves (regression in the linear part of sigmoids). Results presented in Figure 2A are representative of at least three independent experiments performed in duplicate.

These results show that while deletion of the 10, 11, 12 or 13 first residues does not affect hPRL affinity for its receptor (competition curves superimposed), the curve obtained with Δ1-9-hPRL was slightly displaced to the left compared to WT hPRL, representing a small increase of 20% in

affinity, whereas that of  $\Delta 1-14$ -hPRL was displaced to the right, reflecting 2 to 3 fold lower affinity (40% relative affinity).

#### Binding assay of the G129R-containing mutants.

Representative competition curves obtained with the three analogs containing the Gly129→Arg mutation are shown in Figure 2B: WT hPRL (—●—); single mutant G129R-hPRL (—◆—); double mutant  $\Delta 1-9$ -G129R-hPRL (—■—); double mutant  $\Delta 1-14$ -G129R-hPRL (—▲—).

The three curves are displaced to the right by ~1 order of magnitude compared to WT hPRL, reflecting 10 fold lower affinity for the receptor. Averaged from three independent experiments,  $IC_{50}$  were  $166 \pm 47$  ng/ml for  $\Delta 1-9$ -G129R and  $187 \pm 49$  ng/ml for  $\Delta 1-14$ -G129R, compared to  $18 \pm 5$  ng/ml (for WT hPRL). None of the N-terminal deletion improves affinity compared to G129R-hPRL (single mutant).

#### **EXAMPLE 3: BIOACTIVITY OF HPRL ANALOGS.**

##### Experimental protocols

##### Nb2 cell proliferation assay

The reference bioassay for lactogenic hormones is the lactogen-induced proliferation of rat Nb2 lymphoma cells. Rat Nb2 lymphoma cells were obtained from P. W. GOUT (Vancouver, Canada) and cultured as previously described (BERNICHTEIN *et al.*, Endocrinology, 142, 3950-3963, 2001). Nb2 cells were routinely maintained in RPMI 1640 supplemented with 10% HS, 10% heat-inactivated FCS, 2 mM glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and 100 mM  $\beta$ -mercaptoethanol. The proliferation assay was performed as initially described (TANAKA *et al.*, J. Clin. Endocrinol. Metab., 51, 1058-1063, 1980) with minor modifications (BERNICHTEIN *et al.*, Endocrinology, 142, 3950-3963, 2001). Briefly, the assay was performed in 96-well plates using  $2 \times 10^4$  cells/well on starting day, in a final volume of 200  $\mu$ l, including hormones. Cell proliferation was estimated after 3 days of hormonal stimulation by adding 10  $\mu$ l WST-1 tetrazolium salt (ROCHE, Meylan, France). This survival



reagent is metabolized by mitochondria of living cells, which leads to an increase in the OD measured at 450 nm (OD<sub>450</sub>) in a manner that is proportional to the number of cells counted by hemocytometer (BERNICHTEIN *et al.*, Endocrinology, 142, 3950-3963, 2001). The experiments were performed at least three times in triplicate or quadruplicate.

#### Human PRLR transcriptional bioassay (HL5)

Clone HL5 are 293 HEK fibroblasts stably transfected with plasmids encoding the human PRLR and a PRL-responsive reporter gene (containing the sequence encoding the luciferase gene under the control of a six-repeat sequence of the lactogenic hormone response element (LHRE) which is the DNA-binding element of STAT5 (KINET *et al.*, J. Biol. Chem., 274, 26033-26043, 1999).

The HL5 clone was routinely cultured in DMEM-Nut F12 medium supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 700 µg/ml G-418 (clonal selection). The assay was performed in 96-well plates using  $5 \times 10^4$  cells/100 µl/well in medium containing only 0.5% FCS. Cells were allowed to adhere overnight, then 100 µl hormones diluted in FCS-free medium were added to each well. After 24 h of stimulation, cells were lysed (50 µl lysis buffer), then luciferase activity contained in 15 µl cell lysate was counted for 10 sec (BERNICHTEIN *et al.*, Endocrinology, 142, 3950-3963, 2001; KINET *et al.*, J. Biol. Chem., 274, 26033-26043, 1999). To avoid inter-assay variations, all analogs to be compared were systematically tested in the same experiment. In agonism experiments 100 µl of [2x] hormones ("2x" = concentrated 2 times compared to the final concentration required) to be tested are added, whereas in antagonism experiments, a mix of 50 µl of [4x] hormone analogs combined with 50 µl of [4x] WT hPRL (to obtain a final concentration of 1 µg/ml) were added.

#### Ba/F3-hPRLR cell proliferation bioassay

Ba/F3 cells are mouse pro-B lymphoid cells dependent on Interleukin-3 (IL-3) for growth. Ba/F3-hPRLR cells were obtained after transfection using a plasmid

encoding the hPRLR, and a double selection involving G-418 treatment and substitution of hPRL for IL-3 in the growth medium. Cells were transfected (electroporation) using the plasmid encoding the hPRLR and the population stably  
 5 expressing the receptor was selected after G-418 treatment. Ba/F3-hPRLR cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 500-1000 µg/ml G-418, and 10 ng/ml WT-hPRL instead of IL-3. Optimal conditions  
 10 of bioassay (cell number, starvation time and medium, etc) were determined using WT hPRL as ligand, and are the following: before the proliferation assay, cells were starved for 6 hours in 1% FCS RPMI medium (with additives), then distributed in 96 well-plates at a density of  $5 \times 10^4$   
 15 cells/well in a final volume of 100 µl in the same medium (excluding hormones). In agonism experiments, 100 µl of [2x] hormones were added; in antagonism experiments, 50 µl of [4x] hormones to be tested for antagonistic properties and 50 µl of [4x] WT hPRL (final concentration of 10 ng/ml) were added.  
 20 Cell proliferation was monitored after 3 days of hormonal stimulation using 10 µl of WST-1. Experiments were performed at least three times in triplicate or quadruplicate.

## Results

### N-terminal deleted analogs

#### 25 *Agonism*

##### *Nb2 cell proliferation assay*

According to previous reports, monomeric hPRL induces cell proliferation in the classical Nb2 cell proliferation assay with a maximal effect at 1-2 ng/ml.

30 Figure 3A shows cell proliferation in presence of increasing concentrations of hPRL (—●—), Δ1-9-hPRL (—□—) and Δ1-14-hPRL (—△—); Figure 3B shows cell proliferation in presence of increasing concentrations of hPRL (—●—), Δ1-10-hPRL (—\*—), Δ1-11-hPRL (—○—), Δ1-12-hPRL (—□—), Δ1-  
 35 13-hPRL (—◇—).

Dose-response curves for this assay were similar for all mutants ( $\Delta 1-9$ -hPRL  $\rightarrow$   $\Delta 1-14$ -hPRL) and WT hPRL ( $EC_{50}$  ranging from 0.57 to 0.87 ng/ml), indicating that N-terminal deletions do not dramatically alter the mitogenic activity of hPRL in this assay.

*Ba/F3-hPRLR cell proliferation bioassay*

In contrast to the assay with Nb2 cells, the hPRLR-mediated proliferation assay with Ba/F3 cells displayed different mitogenic activities of the analogs. WT hPRL induced growth of this cell population in a dose-dependent manner, with maximal effect at  $\sim 10$  ng/ml, which correlates with the cell selection by substituting 10 ng/ml hPRL for IL-3 in routine culture medium.

Figure 4A shows proliferation of Ba/F3 cells in presence of increasing concentrations of hPRL ( $-\bullet-$ ),  $\Delta 1-9$ -hPRL ( $-\square-$ ) and  $\Delta 1-14$ -hPRL ( $-\triangle-$ ); Figure 4B shows proliferation of Ba/F3 cells in presence of increasing concentrations of hPRL ( $-\bullet-$ ),  $\Delta 1-10$ -hPRL ( $--*--$ ),  $\Delta 1-11$ -hPRL ( $--\circ--$ ),  $\Delta 1-12$ -hPRL ( $--\square--$ ),  $\Delta 1-13$ -hPRL ( $--\diamond--$ ).

The dose-response curves obtained with analogs  $\Delta 1-9$ -hPRL,  $\Delta 1-10$ -hPRL,  $\Delta 1-11$ -hPRL,  $\Delta 1-12$ -hPRL and  $\Delta 1-13$ -hPRL were superimposed to that obtained with hPRL, reflecting no alteration of bioactivity. In contrast, the curve of  $\Delta 1-14$ -hPRL was displaced to the right by  $>1$  log, reflecting significantly altered ability to activate the hPRLR in this assay. All analogs were able to induce a maximal level of cell division provided sufficient hormone concentrations were added in the assay.

*Human PRLR transcriptional bioassay (HL5)*

Data of one typical experiment performed in duplicate and representative of three experiments are shown in Figure 5 which depicts hPRL transcriptional activity (% of activity vs WT hPRL maximal effect referred as 100%) in presence of increasing concentrations ( $\mu$ g/ml) of: hPRL ( $-\bullet-$ ),  $\Delta 1-9$ -hPRL ( $-\square-$ ),  $\Delta 1-14$ -hPRL ( $-\triangle-$ ),  $\Delta 1-10$ -hPRL ( $--*--$ ),  $\Delta 1-11$ -hPRL ( $--\circ--$ ),  $\Delta 1-12$ -hPRL ( $--\square--$ ),  $\Delta 1-13$ -hPRL ( $--\diamond--$ ).

The results are expressed in fold induction of luciferase activity (i.e. percentage of activity vs WT hPRL maximal effect referred as 100%).

5        Analogs  $\Delta$ 1-9-hPRL,  $\Delta$ 1-10-hPRL,  $\Delta$ 1-11-hPRL,  $\Delta$ 1-12-hPRL and  $\Delta$ 1-13-hPRL were undistinguishable in this assay, with curves displaced to the left compared to WT hPRL ( $EC_{50}$  decreased by ~2 fold). In addition, the maximal response induced by all these analogs was higher compared to WT hPRL (120-140%), reflecting super-agonistic properties.

10        In contrast,  $\Delta$ 1-14-hPRL was less active than hPRL, regarding both its dose-response curve ( $EC_{50}$  ~3-fold higher) and its maximal activity (60 % of WT hormone).

#### Antagonism

15        In agreement with their intrinsic agonistic activity, none of the N-terminal deletion mutants displayed antagonistic activity in any of the three bioassays used in this study (data not shown).

#### Double mutants

20        All experiments involve the single mutant (G129R-hPRL) and the double mutants  $\Delta$ 1-9-G129R-hPRL and  $\Delta$ 1-14-G129R-hPRL.

#### Nb2 cell proliferation assay

25        AGONISM. Figure 6 shows cell proliferation without hPRL ( $\square$ ) and in presence of increasing concentrations of purified WT hPRL ( $\blacksquare$ ), G129R-hPRL ( $\square$ ),  $\Delta$ 1-9-G129R-hPRL ( $\equiv$ ) and  $\Delta$ 1-14-G129R-hPRL ( $\boxtimes$ )

30        WT hPRL induces maximal proliferation at 1-2 ng/ml, whereas the dose-dependent mitogenic effect of G129R-hPRL is shifted to the high concentrations, but reaches (sub) maximal proliferation. In contrast, both double N-Terminal deleted mutants  $\Delta$ 1-9-G129R-hPRL and  $\Delta$ 1-14-G129R-hPRL are devoid of significant agonistic activity.

35        As previously reported (GOFFIN et al., J. Biol. Chem., 269, 32598-32606, 1994; BERNICHTEIN et al., Endocrinology, 142, 3950-3963, 2001), the agonistic dose-response curve obtained with G129R-hPRL is shifted by more than two log units to the right compared to WT hPRL, with

maximal effect achieved at about 0,5 to 1 µg/ml. Interestingly, this agonistic activity is totally abolished when N-terminal tail of G129R-hPRL is deleted (meaning in Δ1-9-G129R-hPRL and Δ1-14-G129R-hPRL analogs), and this was true even at concentrations up to 4 orders of magnitude higher than the concentration leading to maximal activity of WT hPRL (1 ng/ml vs 10 µg/ml).

ANTAGONISM. Antagonistic assays performed using Nb2 cells failed to display any inhibition of WT hPRL, whatever the analog tested. The reason for this result is under investigation.

*Human PRLR transcriptional bioassay (HL5)*

AGONISM. Figure 7A shows activation of the LHRE-luciferase reporter gene by increasing concentrations of WT hPRL (■), and the three G129R-containing analogs, G129R-hPRL (□), Δ1-9-G129R-hPRL (≡), and Δ1-14-G129R-hPRL (▣).

The agonistic activity of G129R-hPRL is extremely reduced in this assay, reaching a maximal level <2% of hPRL activity. Similarly, none of the double mutant induced detectable level of luciferase activity, even when tested at extremely high concentrations (up to 50 µg/ml).

ANTAGONISM. The results are shown in Figure 7B: Δ1-9-G129R-hPRL (-■-), Δ1-14-G129R-hPRL (-▲-), G129R-hPRL (-◆-).

In agreement with their relative affinity for the hPRLR, the antagonistic properties of the three analogs were very similar, but repeatedly showed the following order of activity: G129R-hPRL > Δ1-9-G129R-hPRL > Δ1-14-G129R-hPRL.

*Ba/F3-hPRLR cell proliferation bioassay*

AGONISM. Figure 8A shows cell proliferation in presence of increasing concentrations of purified WT hPRL (■), G129R-hPRL (□), Δ1-9-G129R-hPRL (≡), and Δ1-14-G129R-hPRL (▣).

Maximal effect of WT hPRL is obtained at 10 ng/ml. G129R-hPRL induced sub-maximal proliferation with a dose-response curve displaced by 2 logs to the high

concentrations. In contrast, none of the double mutants ( $\Delta 1-9$ -G129R-hPRL and  $\Delta 1-14$ -G129R-hPRL) induced significant proliferation.

As in the Nb2 assay, the curve obtained for G129R-hPRL was displaced to the right by ~2 log units and achieved sub-maximal (50-80%) level compared to hPRL. At high concentrations, hPRL and G129R-hPRL displayed bell-shaped curves, a typical observation when using these ligands (KINET *et al.*, Recent Res. Devel. Endocrinol., 2, 1-24, 2001). Both  $\Delta 1-9$ -G129R-hPRL and  $\Delta 1-14$ -G129R-hPRL failed to display any agonistic activity, even at concentration as high as 10  $\mu$ g/ml.

ANTAGONISM. Antagonistic assays were performed by competing a fixed concentration of WT hPRL (10 ng/ml) with increasing concentrations of the analogs. Figure 8B shows cell proliferation in presence of increasing concentrations of  $\Delta 1-9$ -G129R-hPRL ( $-\blacksquare-$ ),  $\Delta 1-14$ -G129R-hPRL ( $-\blacktriangle-$ ), G129R-hPRL ( $-\blacklozenge-$ ) competing with the fixed concentration of WT hPRL.

The three mutants in which Arg is substituted for Gly129 (G129R-hPRL,  $\Delta 1-9$ -G129R-hPRL and  $\Delta 1-14$ -G129R-hPRL) displayed similar antagonistic activities, meaning that efficient competition with WT hPRL required high molar excess of the analog being used (10 to 50 fold), irrespective of N-terminal deletions. With respect to the double mutants, the competitive inhibition of WT hPRL-induced activity presumably reflects a true phenomenon of antagonism, since these analogs are devoid of intrinsic agonistic effect (Figure 8A). In contrast, since G129R-hPRL displays a significant agonistic activity, the inhibitory effect observed in competition assays presumably reflects a combination of real antagonism and self-antagonism phenomenon (GOFFIN *et al.*, J. Biol. Chem., 269, 32598-32606, 1994; BERNICHTEIN *et al.*, Endocrinology, 142, 3950-3963, 2001, KINET *et al.*, Recent Res. Devel. Endocrinol., 2, 1-24, 2001).

## CLAIMS

1) An antagonist of a mammal prolactin receptor, wherein said antagonist is a variant of mammal prolactin having the following mutations:

- 5 a) a mutation or set of mutations within the 14 N-terminal amino acids, wherein said mutation or set of mutations prevents the formation of the disulfide bridge between Cys<sub>4</sub> and Cys<sub>11</sub>, and  
b) a sterically hindering mutation or set of mutations within  
10 binding site 2 of prolactin.

2) A variant of prolactin according to claim 1, wherein mutation(s) a) comprise the deletion of at least the 4 N-terminal residues of prolactin.

- 3) A variant of prolactin according to claim 2, wherein mutation(s) a) comprise the deletion of the 9 N-terminal residues of prolactin.  
15

4) A variant of prolactin according to claim 3, having the following mutations:

- a deletion of at least the 9 N-terminal residues and up to the 14 N-terminal residues;  
20 and  
- a G129R substitution.

5) A variant of prolactin according to any of claims 1 to 4, which is a variant of human prolactin.

- 25 6) A polynucleotide encoding a variant of prolactin of any one of claims 1 to 5.

7) An expression cassette comprising a polynucleotide of claim 6.

- 8) A recombinant vector comprising a polynucleotide of claim 6.  
30

9) An host cell transformed by a polynucleotide of claim 6.

10) A transgenic non-human mammal transformed with a polynucleotide of claim 6.

- 35 11) A therapeutic composition comprising or a polynucleotide of claim 6.

12) Use of a variant of prolactin according to any of claims 1 to 5, or of a polynucleotide of claim 6 for obtaining a therapeutic composition for treating or preventing a disease involving PRL- mediated effects.



#### ABSTRACT

The invention relates to mammal prolactin (PRL) variants having a mutation or set of mutations within the 14 N-terminal amino acids thereby preventing the formation of a disulfide bridge between Cys<sub>4</sub> and Cys<sub>11</sub>, and a sterically hindering mutation or set of mutations within binding site 2 of PRL. These variants are useful as antagonists of mammal prolactin receptors (PRLR), more particularly of human prolactin receptor (hPRLR).

wt hormones

hPRL

hGH

.....F PTIPLSRLEFDNAMLRAHRLHQLAFTYQ

1 9 14 Helix 1

LPICPGGAAR CQVTLRDLEFDRVVLSHYIHNLSSEMF

## hPRL mutants

**$\Delta 1-9-hPRL$**

**$^{10}\text{R}$  SQVTLRDLEFDRAVLSHYIHNLSEMF**

**$\Delta 1-10-hPRL$**

**11 SÖVTLRDLEDRÄVLSHYIHNLSSEMS**

**$\Delta 1-11-hPRL$**

**<sup>12</sup>QVTLRDRAVLSHYIHNLSEMF**

# $\Delta 1-12-hPRL$

**<sup>13</sup>VTLRDLFDRAVLSHYIHNLSSEMF**

**Δ1-13-hPRL**

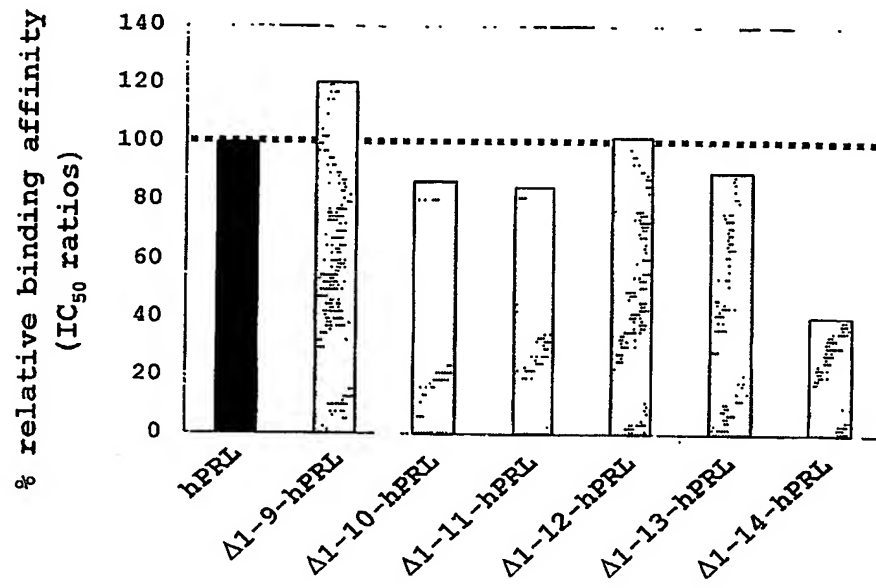
**<sup>14</sup>TLRDLEDRVLSHYIHNLSEMF**

**$\Delta 1-14-hPRL$**

**15 L R D L F D R A V L S H Y I H N L S E M F S**

**FIG. 1**

A.



B.

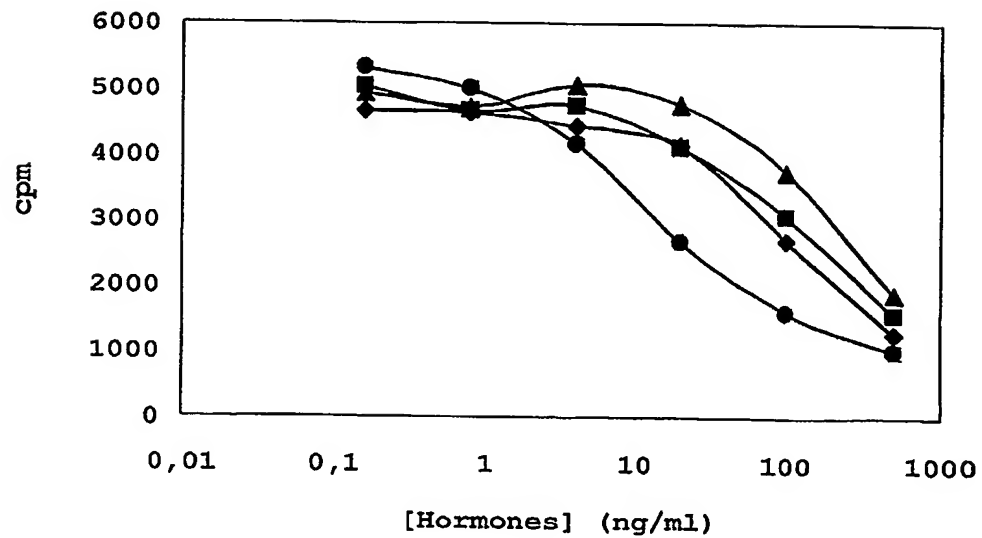
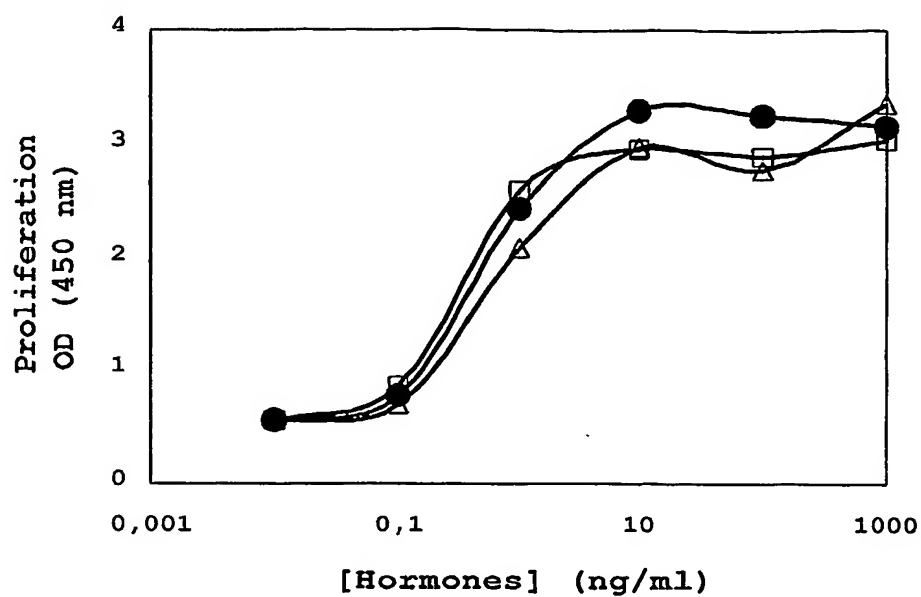
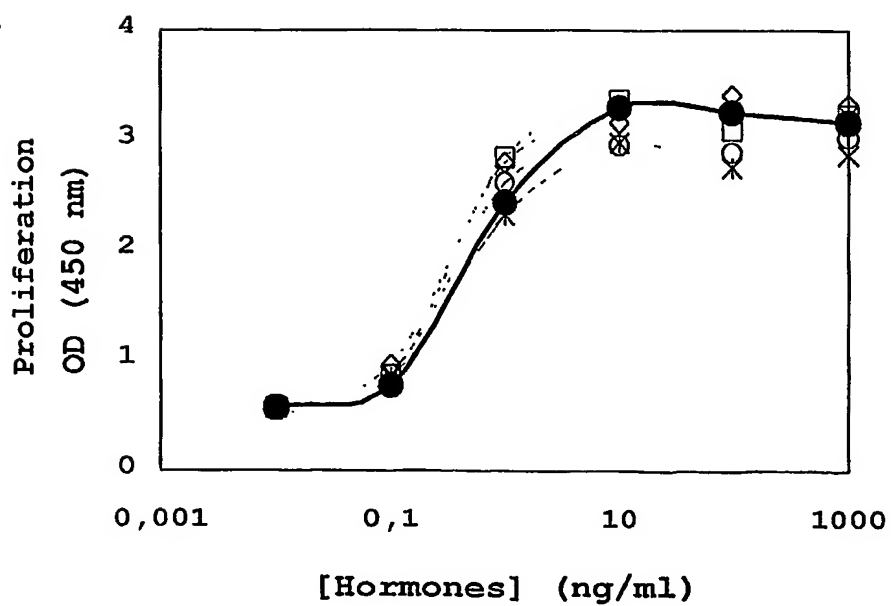
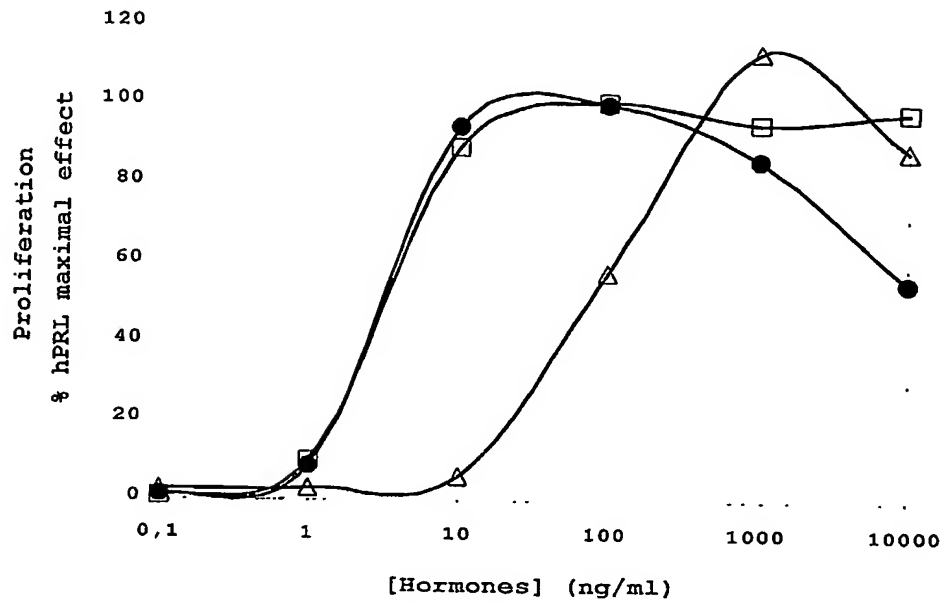


FIG. 2

**A.****B.****FIG. 3**

A.



B.

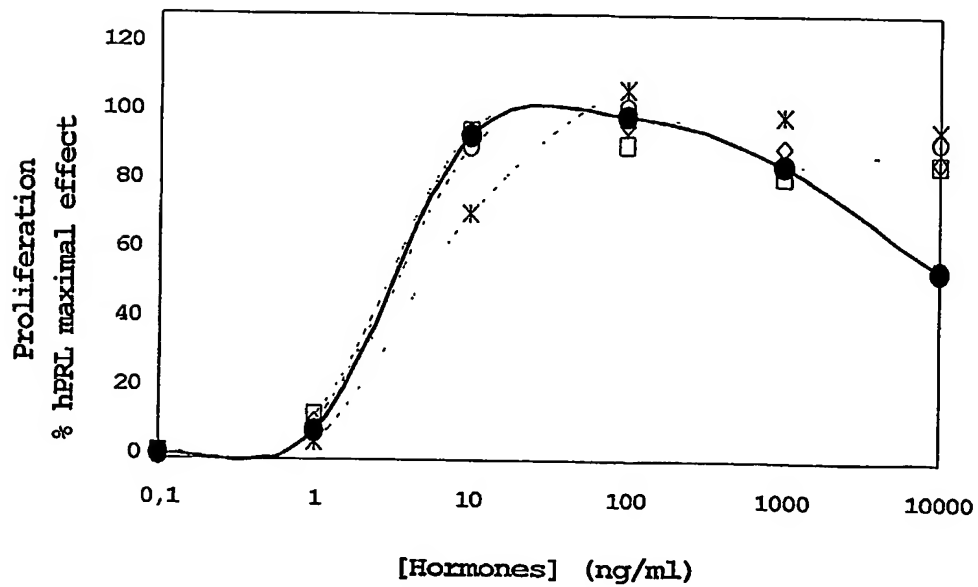


FIG. 4

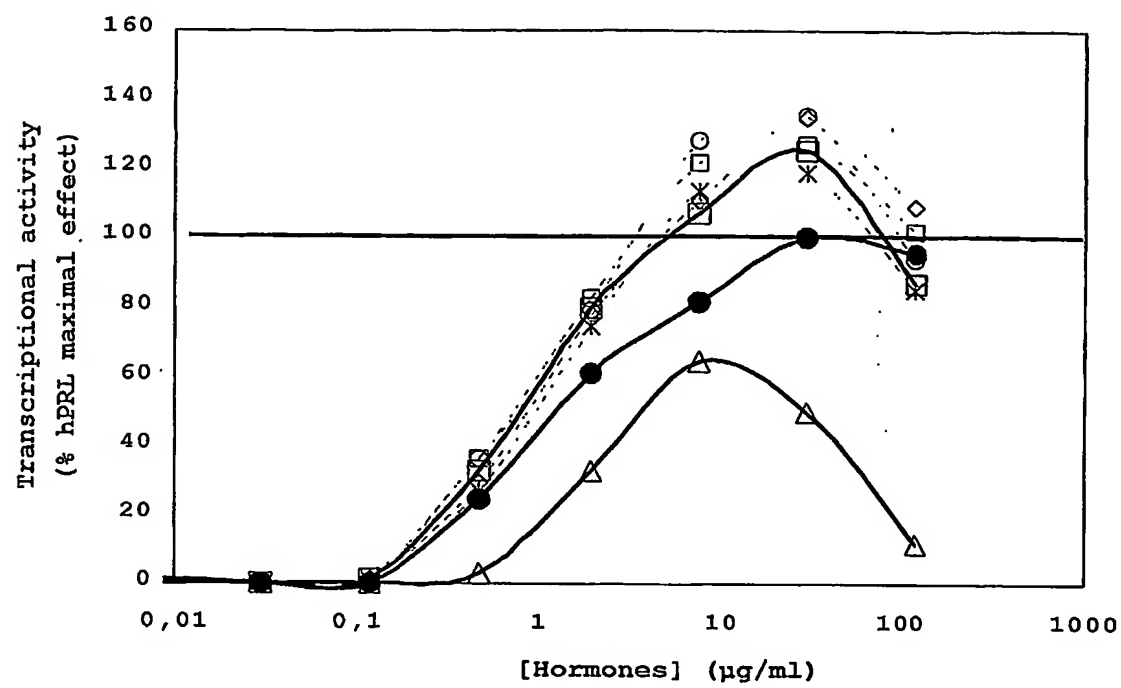
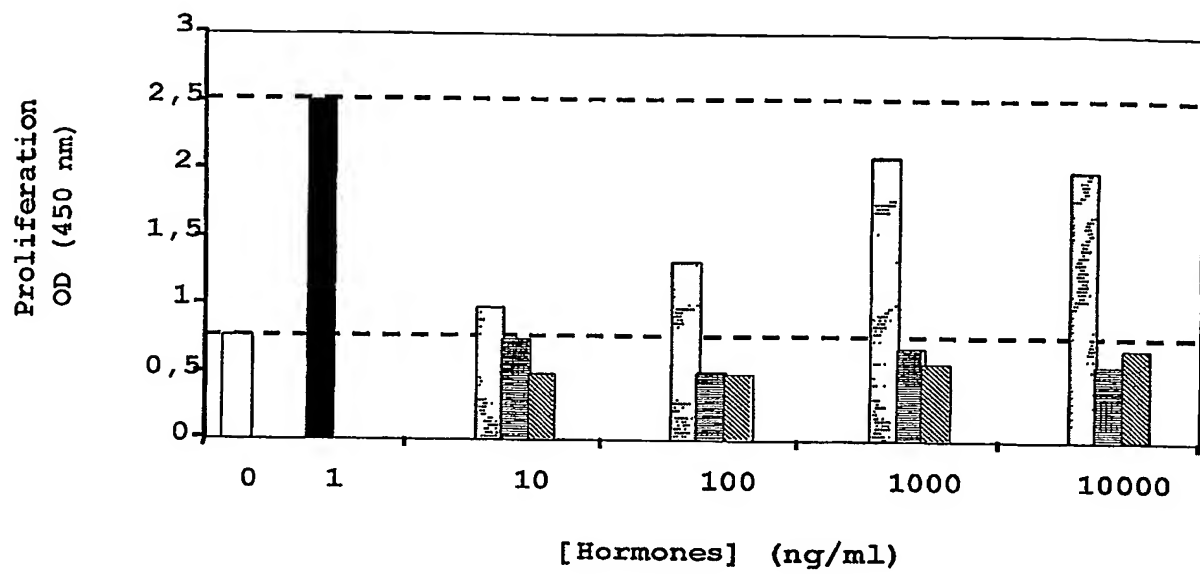
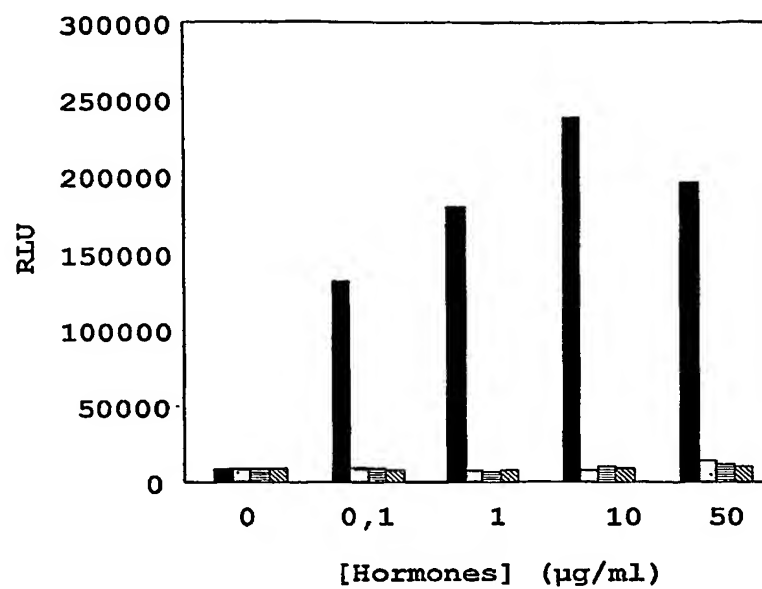
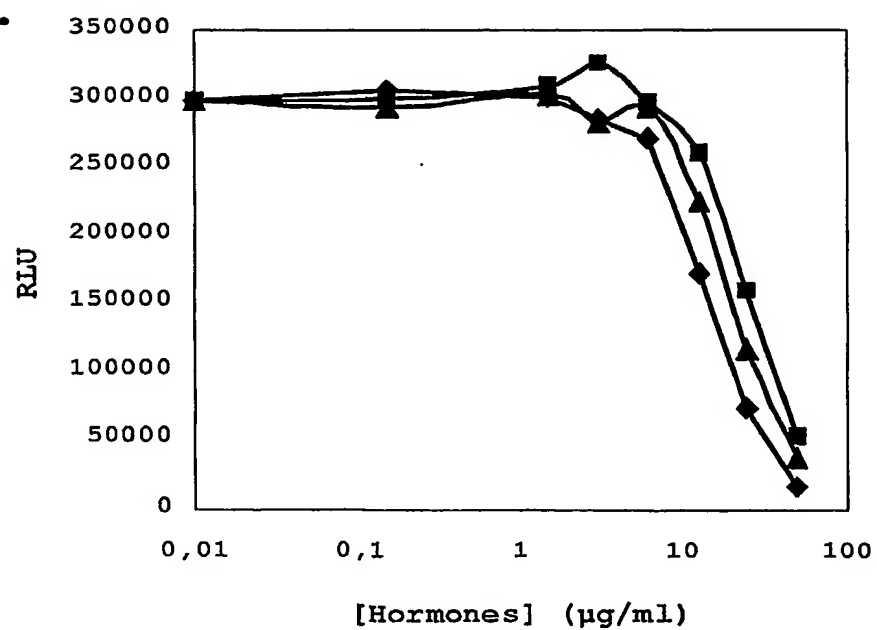
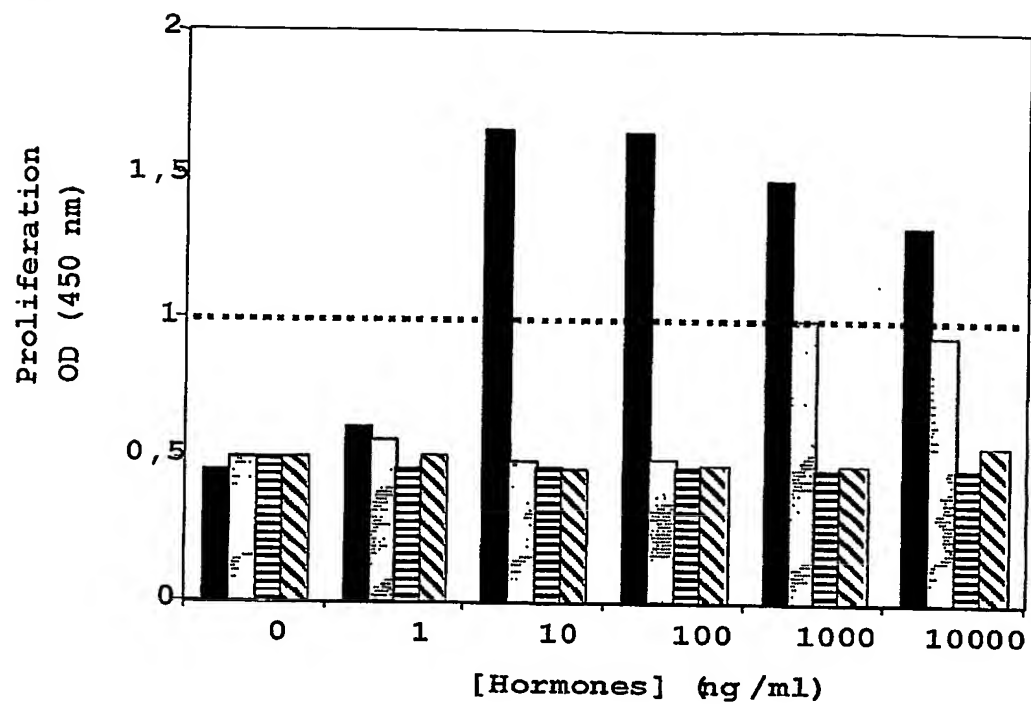
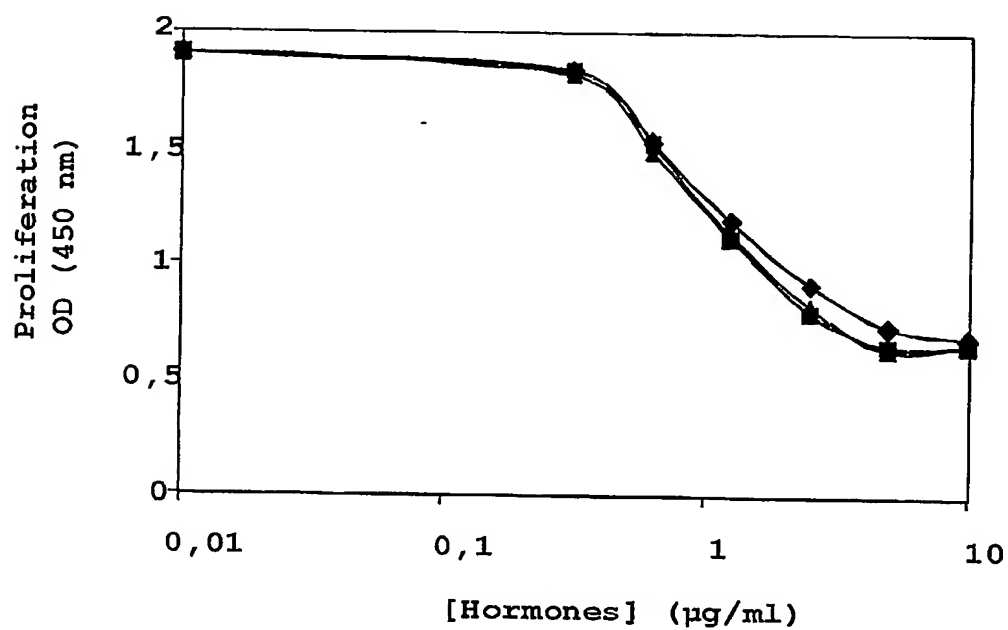


FIG. 5

**FIG. 6**

**A.****B.****FIG. 7**



**A.****B.****FIG. 8**

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